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Role of IRS1 and IRS2 in Modulating ErbB-induced Tumorigenesis

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Previous studies suggested that the adapter proteins IRS1 and IRS2 may interact with ErbB1 or ErbB2 receptors. However, I found that overexpression of IRS2 didn't affect ErbB2-induced tumorigenesis or metastasis. Supporting this, I failed to find an association between ErbB2 and IRSs in breast cancer cells. I thus expanded my studies to the IGF-IR receptor for which IRSs serve as the main adapter proteins for downstream signaling. I performed reverse phase proteomic analysis of IGF-I and insulin action in 23 breast cancer cell lines. We found that IRS-1 is a central signaling node in the IGF-IR signaling pathways, and it needs to be inhibited for optimal pathway blockade. Finally, I studied the role of an IGF-I gene signature in predicting response to an IGF-IR inhibitor. I confirming that this signature can measure IGF activity, as it was reversed in three different models (cancer cell lines or xenografts) treated with three different anti-IGF-IR therapies. We found that the IGF signature is present in triple negative breast cancer (TNBC) cell lines, which were especially sensitive to an IGF-IR tyrosine kinase inhibitor (BMS-754807). Consistent with this, comparative gene expression analysis among the most resistant and sensitive cell lines identified 114 differentially expressed genes which confirmed TNBC as being sensitive. Furthermore, sensitivity to BMS-754807 significantly correlated to expression of the IGF gene signature. Supporting a role for IGF-IR signaling in TNBC, the primary human TNBC tumorgraft MC1 showed high levels of IGF-IR expression, activity, and IGF gene signature score, and showed growth inhibition following treatment with BMS-754807 as a single agent, and in combination with docetaxel tumor regression occurred until no tumor was palpable. This regression was associated with reduced proliferation, increased apoptosis, and mitotic catastrophe. These studies have further highlighted a critical role for IRSs in IGF-IR action in breast cancer.

15. SUBJECT TERMS
Insulin Receptor Substrate (IRS), IRS1, IRS2, IGF-IR, triple-negative breast cancer, BMS-754807, IGF, small molecule inhibitor

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INTRODUCTION

Insulin receptor substrate 1 and 2 (IRS1 and IRS2) are adapter proteins that link signaling from upstream activators to multiple downstream effectors. IRSs modulate and coordinate multiple signaling cascades involved in normal growth, metabolism and survival suggesting that they may play a role in cancer. Indeed, IRSs are required for the transforming ability of many oncogenes and IRSs are elevated and hyperactive in many human tumors and high IRS1 levels are associated with poor prognosis in breast cancer. Adapter proteins have been shown to play an important role in epidermal growth factor receptor (ErbB2/Her2/Neu) amplified breast cancer. However, there is little known about the IRS interaction with ErbB2 in cancer development and progression. **Therefore, I hypothesized that ErbBs bind and phosphorylate IRSs, and that levels of IRSs will modulate ErbB-induced tumorigenesis**.

During the first year of my progress report I addressed this hypothesis by examining the role of IRS2 in ErbB2-mediated tumorigenesis in transgenic mice. I crossed MMTV-ErbB2 and MMTV-IRS-2 mice, however I found that elevated IRS2 had no effect upon ErbB2 induced tumorigenesis. I also examined a putative interaction between ErbB2 and IRS1 and/or IRS2, however, I was unable to conclusively show this association in either mammary epithelial cells, or breast cancer cells (detailed in my first annual report). I therefore officially changed my statement of work to expand upon the role of IRSs in IGF-IR signaling in breast cancer.

IGF-IR is a major regulator of growth, survival, and migration and invasion. Once the ligands IGF-I or IGF-II bind to IGF-IR, it activates a cascade of downstream signaling events, the best characterized are the ERK1/2 and phosphatidylinositol-3'-kinase (PI3K) pathways (1).

IGF-IR is oncogenic, transforming NIH3T3 or mouse embryonic fibroblast (MEF) cells (2). We showed that overexpression of a constitutively active IGF-IR in the mouse mammary gland resulted in rapid mammary tumorigenesis (3), and this was then supported by a similar observation using inducible overexpression of wild-type IGF-IR (4). We subsequently showed that constitutive IGF-IR can transform immortalized mammary epithelial cells (MCF10A), and that growth of IGF-IR transformed mammary epithelial cells was blocked *in vitro* and *in vivo* by a new IGF-IR tyrosine kinase inhibitor (BMS-536924) (5). This same inhibitor was found to be active against many breast cancer cell lines, and was found to restore apical/basal polarity to MCF7 breast cancer cells grown in matrigel (6).

In breast cancer specimens, IGF-IR is detected at very high frequency and is overexpressed compared to normal breast and high serum IGF-I levels predict increased breast cancer risk (1). Numerous agents targeting IGF-IR have recently entered clinical trials, and early results are very encouraging (7). Two different classes of anti-IGF-IR inhibitors are in development: monoclonal antibodies which are highly specific for IGF-IR and cause downregulation of the receptor, and tyrosine kinase inhibitors which often cross-react and block insulin receptor (InsR) as well as IGF-IR action (7).

The significance of this research project is the realization that IRSs are not simply mitogenic and metabolic signaling elements, but that they have numerous other functions that strongly implicate them in cancer development and progression. I believe that this research project will increase our understanding of IRSs in modulating its upstream receptors in cancer development and progression, and may provide evidence and strategies for inhibiting IRSs as a therapeutic strategy in breast cancer.

BODY

1) Research and Training Accomplishments

The Breast Center at Baylor College of Medicine (BCM) provided a unique training environment for my graduate studies with multiple opportunities for me to grow as a young research scientist. In the past three years, I have taken full advantage of these opportunities and will outline my primary accomplishments here:

- attended and presented data in poster format at the Department of Medicine Research Symposia (April 2008)
- attended and presented data in poster format at the Gordon Conference on " Insulin-like growth factors in physiology and disease" (March 2009)
- audited a Translational Breast Cancer Research course taught here at BCM by faculty members of the Breast Center (December 2008)
- contributed a section to a text book chapter entitled "Insulin-Like Growth Factor Signaling in Normal Mammary Gland Development and Breast Cancer Progression," which was published in 2008.
- published a first author research article entitled " BMS-536924 Reverses IGF-IR-Induced Transformation of Mammary Epithelial Cells and Causes Growth Inhibition and Polarization of MCF7 Cells".
    - **Litzenburger BC**, Kim H J, Kuiatse I, Carboni J M, Attar R M, Gottardis M M, Fairchild C R, Lee AV. BMS-536924 reverses IGF-IR-induced transformation of mammary epithelial cells and causes growth inhibition and polarization of MCF7 cells. *Clin Cancer Res.* 2009 Jan 1;15(1):226-37. PMID: 19118050
- attended and presented data in a talk at the Sue and Lester Smith Breast Center Retreat (September 2009). I won the prize for best Oral presentation.
- attended and presented data in poster format at the Dan Duncan Cancer Center Symposium (November 2009)
- attended and presented data in poster format at the San Antonio Breast Cancer Symposium. For the work presented in this poster I received the San Antonio Breast Cancer Symposium Basic Science Scholarship (December 2009)
- presented data in a talk at the Breast Disease Seminar (February, 2010)
- submitted a first author research article entitled " High IGF-IR activity in triple-negative breast cancer correlates with sensitivity to anti-IGF-IR therapy" to Clinical Cancer Research. Manuscript had favorable reviews and was resubmitted with minor corrections.
    - **Litzenburger BC**, Creighton CJ, Tsimelzon A, Chan BT, Hilsenbeck SG, Wang T, Carboni J M, Gottardis M M, Huang F, Chang, Lewis M T, Rimawi M F, Lee A V. High IGF-IR activity in triple-negative breast cancer cell lines and tumorgrafts correlates with sensitivity to anti-IGF-IR therapy. In revision *Clin Cancer Res* 2010
- Defended my thesis in June 2010 and was awarded the PhD.

2) Research Project

During the first year of the project, I mainly focused on the role of IRS2 in ErbB2-mediated tumorigenesis in transgenic mice. I found that:

- Overexpression of IRS2 did not alter branching morphogenesis in adult ErbB2 overexpressing mice.
- Overexpression of IRS2 had no affect upon ErbB2-induced mammary tumorigenesis, with median time to tumor formation of 30.5 weeks, respectively.
- We observed similar rates of lung metastasis in MMTV-ErbB2 mice compared to ErbB2/IRS2 bigenic mice.
- Immunoprecipitation failed to show an interaction between ErbB2 and IRSs in both mammary epithelial cells and breast cancer cells (this was based upon a preliminary literature suggesting there would be an interaction).
- Overexpression of ErbB2 didn't affect the activation status of IRSs.

The results of the first year showed that we could not clearly decipher the role of insulin receptor substrates (IRSs) in ErbB2 induced tumorigenesis *in vivo*. We submitted a revised statement of work for year 2 and 3 to expand our research project to other receptors such as IGF-IR for which IRSs serve as the main adapter proteins for downstream signaling. The new approved aims were:

Aim 1. Test whether IRSs are critical to ErbB receptor signaling and function

i) Perform transient or stable knockdown of IRS1 or IRS2 levels using short-term si/shRNA in mammary cancer cells derived from MMTV-ErbB2 transgenic mice and examine effects on signaling downstream of both IGF-IR and ErbB receptors. (Months 12-15).

ii) Perform transient or stable knockdown of IRS1 or IRS2 levels using short-term si/shRNA in MMTV-ErbB2 mammary cancer cells and examine effects on acini morphogenesis in three-dimensional culture and growth in soft agar (Months 12-16).

iii) Determine the biological effect of IRS1 and IRS2 downregulation in the above model by examination of proliferation, apoptosis, and polarity in three-dimensional culture. (Months 14-20).

Aim 2. Examine the importance of IRS levels on response of breast cancer cells and mammary epithelial cells to IGF-IR signaling (Months 12-24)

i) Correlate levels of IRSs with response to IGF-I and insulin in a large panel of breast cancer cell lines. (Months 12-18).

ii) Determine the effect of loss of IRS-1 and IRS-2 on IGF-IR disrupted mammary acini formation (CD8-IGF-IR-MCF10A cells) (Months 15-20).

iii) Test the effect of IRS knockdown on IGF-IR-mediated epithelial to mesenchymal transition and transformation (Months 18-24).

Aim 3. Identify biomarkers for response to IGF-IR and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor (Months 18-36)

i) Use transcriptomic profiling of a large panel of breast cancer cell lines to identify markers of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor (Months 18-24).

ii) Test whether response of cells to the IGF-IR inhibitor is correlated with markers identified in Aim i (Months 24-30).

iii) Examine if an IGF-IR inhibitor is effective in breast cancer xenografts identified as having an active IGF-IR pathway (Months 24-36).

During my thesis, the majority of my work and success (and subsequent publication) came from Specific Aim 3. In Specific Aim 2 I participated in a large proteomic effort which is just being written up by another student in Dr Lee's laboratory (with me as co-author). Specific Aim 1 turned out to be technically challenging and is being continued in Dr. Lee's laboratory since my departure. I will now describe the results from each of the aims.

Specific Aim 1. **Test whether IRSs are critical to ErbB2 receptor signaling and function**

i) Perform transient or stable knockdown of IRS1 or IRS2 levels using short-term si/shRNA in mammary cancer cells derived from MMTV-ErbB2 transgenic mice and examine effects on signaling downstream of both IGF-IR and ErbB receptors. (Months 12-15).

We isolated mammary epithelial tumor cell lines from MMTV-ErbB2 transgenic tumors and immunoblotted these to show that they expressed ErbB2 and IRS2. They also expressed IRS1 (data not shown).

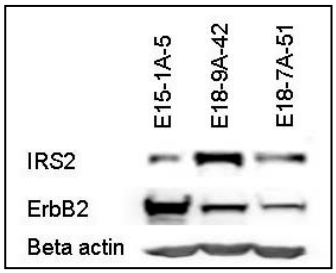


Figure 1: MMTV-ErbB2 mouse cell lines express ErbB2 and IRS2. The mouse cell lines E15-1A-5, E18-9A-42, E18-7a-51 were cultured in complete medium. Protein levels were detected by immunoblot analysis using antibodies against IRS2 and ErbB2 or beta actin.

We had previously used used Dharmacon siRNA pools to knockdown human IRS1 and IRS2 in MCF-10A cells with good success. Unfortunately the knockdown of mouse IRS-1 and IRS-2 using species specific siRNA was insufficient to reduce IRS levels for further study.

ii) Perform transient or stable knockdown of IRS1 or IRS2 levels using short-term si/shRNA in MMTV-ErbB2 mammary cancer cells and examine effects on acini morphogenesis in three-dimensional culture and growth in soft agar (Months 12-16).
Not performed due to the lack of knockdown in 1.i

iii) Determine the biological effect of IRS1 and IRS2 downregulation in the above model by examination of proliferation, apoptosis, and polarity in three-dimensional culture. (Months 14-20).
Not performed due to the lack of knockdown in 1.i

Despite the lack of success in this Aim, the Lee laboratory continues to study the role of IRSs in ErbB function, and is now using shRNA to knockdown IRSs for future studies.

Specific Aim 2. **Examine the importance of IRS levels on response of breast cancer cells and mammary epithelial cells to IGF-IR signaling**

- *Correlate levels of IRSs with response to IGF-I and insulin in a large panel of breast cancer cell lines*

To accomplish this aim we have collaborated with Dr Gordon Mills at UT MD Anderson Cancer Center using the high-throughput reverse phase protein arrays (RPPA) assay (8, 9). In this assay, up to 1000 protein lysates are printed with five serial dilutions in duplicate on a glass slide (max 12,000 spots) and then each slide probed with an antibody. More than 150 antibodies are validated for RPPA. We first started with an initial dose-finding pilot study for IGF-I and insulin. MCF7 cells were stimulated for 15mins with increasing doses of IGF-I and insulin, lysed and interrogated with over 100 antibodies by RPPA (including IGF-IR, p-IGF-IR, and IRSs). The experiment was successful and we noted interesting differences in these signaling pathways.

Subsequently, to correlate levels of IRSs with response to IGF-I and insulin in a large panel of cell lines, we repeated the experiment in 23 breast cancer cell lines, starved them overnight in SFM, and then incubated with 10nM IGF-I or 10nM insulin over a time course (0, 5min, 10min, 30min, 6hr, 24hr, 48hr). This was done in triplicate for a total of 621 lysates which were printed for RPPA analysis (over 150,000 data points). Bioinformatic analysis of this data is underway, however we have many interesting findings. We first produced heatmaps of the changes in protein expression or phosphorylation and noted three different types of response 1) MCF-7 like, 2) HER2-like, and 3) minimal to no response (data now shown). We collaborated with a systems biologists (Prahlad Ram,UT MD Anderson Cancer Center) to develop algorithms which could model the response in the cell lines. Figure 2 shows response for 5 protein markers in MCF-7 stimulated with IGF-I. The black dots are the raw data from the RPPA and the red line is the computer modeled simulation of the data using ordinary differential equations (ODE) with mass particle swarm optimization.

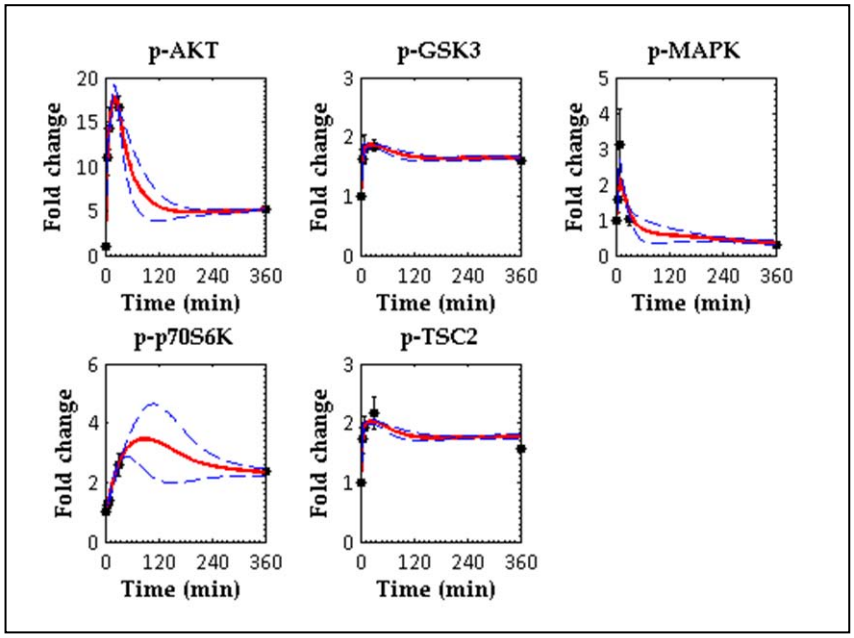


Figure 2: Time dependent regulation of IGF-I signaling in MCF-7 cells. Graphs depict the change in phosphorylation status of 5 proteins following IGF-I stimulation of MCF-7 cells for increasing time. Black dots represent the raw data from the RPPA array and the red line represents a computer simulation of the data (blue dashed line is confidence intervals).

Dr Prahlad Ram's group then used the computer simulation to predict what would happen to each component in the IGF-IR signaling network (including IRS-1) when each component is inhibited. Excitingly, this analysis showed that blockage of many pathways led to upregulation of IRS-1 phosphorylation and activity, and that IRS-1 is a major component that needs to be silenced in breast cancer cells. This is shown in Figure 3, where inhibition of proteins on the X-axis results in changes in proteins listed on the Y-axis. Note that in the two cell lines shown, loss

of IRS-1 has a major impact upon all signaling molecules in the IGF pathway, indicating that IRS1 is a critical signaling node in this pathway.

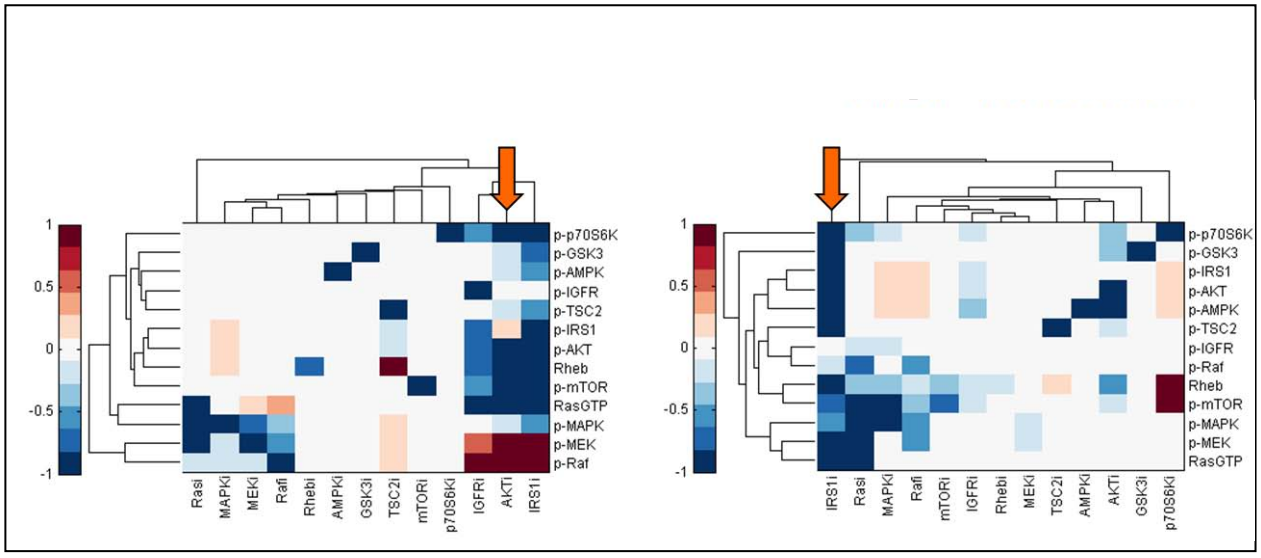


Figure 3. Computer modeling of inhibitor action in MCF7 and MCF-10A cells. The effect of single-molecule inhibition on IGFR signaling in MDA-MB231 cells. Differential levels of proteins in single molecule–inhibited versus non-inhibited cells after stimulation with 50 ng/mL IGF-1 were predicted using the trained mass action model. Numerical values were converted to $\log_{10}$. Blue, inhibition; white, no variation; red, activation. Inhibited proteins are on the X-axis and the effect on other proteins is on the Y-axis. Note that IRS-1 shows that greatest effect in both cell lines (orange arrow in MCF10A). In MCF7 cells, inhibition of Akt (orange arrow) and IGF-IR is also effective.

- *Determine the effect of loss of IRS-1 and IRS-2 on IGF-IR disrupted mammary acini formation (CD8-IGF-IR-MCF10A cells)*
  This was not performed due to the expansion and development of Aim 3.

- *Test the effect of IRS knockdown on IGF-IR-mediated epithelial to mesenchymal transition and transformation*

This was not performed due to the expansion and development of Aim 3.

Specific Aim 3. **Identify biomarkers for response to IGF-IR and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor**

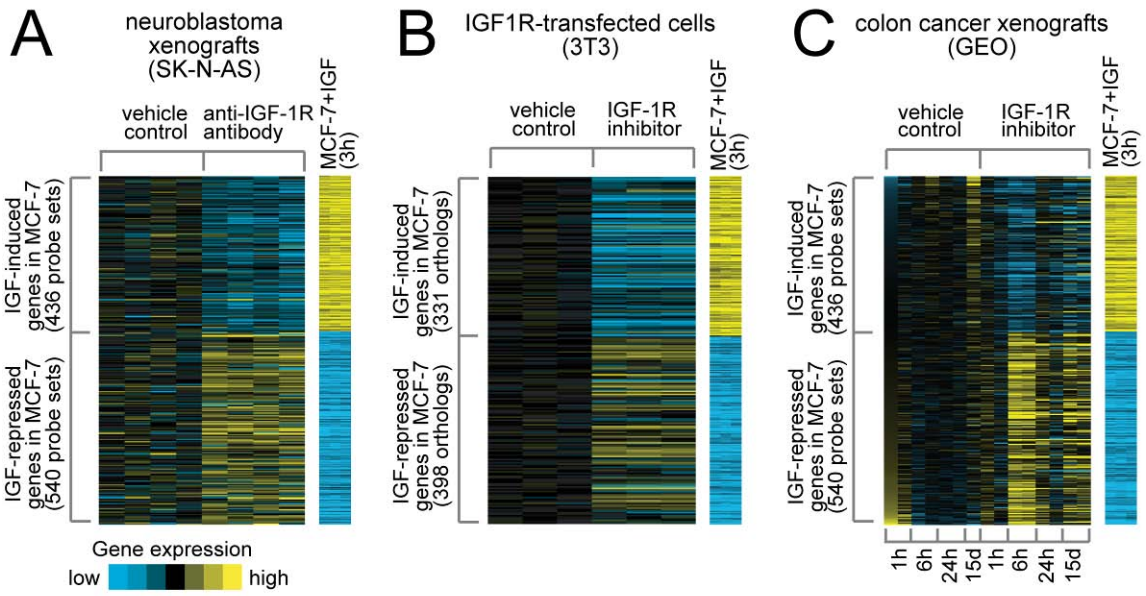
1. *Use transcriptomic profiling of a large panel of breast cancer cell lines to identify markers of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor*

The lack of biological effect of IRS2 on ErbB2 induced tumorigenesis in the transgenic mice led us to expand our research project to the IGF-IR receptor for which IRSs serve as the main adapter proteins for downstream signaling. Experimental studies *in vitro* and *in vitro* have provided substantial evidence for a role of IGF-IR in human breast cancer. Overexpression of a constitutively active IGF-IR or inducible overexpression of wild-type IGF-IR in the mouse

mammary gland results in rapid mammary tumorigenesis (3, 4). Consistent with this, overexpression of IGF-IR transforms immortalized mammary epithelial cells (MCF10A) (5, 10, 11). Clinical studies support the importance of IGFs in breast cancer. In breast cancer specimens, IGF-IR is detected at very high frequency and is overexpressed compared to normal breast. In addition, high serum IGF-I levels predict increased breast cancer risk (7).

To identify markers for response to IGF-IR therapy and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor we used a biased and an unbiased approach. In the biased approach we used an 'IGF-I gene signature' pattern of genes up- or down-regulated by IGF-I that we previously reported (12). The IGF-I gene signature was derived from MCF7 cells that were starved over night and then stimulated with IGF-I for 3 hours and 24 hours. RNA was isolated and microarray analysis was performed. Only genes modulated by IGF-I at both time points were included in the IGF-I gene signature. In addition, we attempted to reduce the importance of proliferative genes by 1) removing genes annotated as being associated with proliferation in Gene Ontology (GO), and 2) removing genes that were found to be induced in fibroblasts stimulated to proliferate by serum (12). In our previous report we found that this IGF signature was present in human breast cancers, specifically the subtypes luminal B and TNBC (12). Triple-negative breast cancers (TNBC) are characterized by low to absent expression of ER, progesterone receptor (PR) and HER-2 (13, 14) and account for up to 20-25% of all breast cancers. TNBC currently has no targeted therapies, and often responds poorly to chemotherapy (15). TNBC preferentially affects younger women and African-American women, and is associated with high histological grade and aggressive clinical behavior (16).

We first set out to examine the effect of IGF-IR inhibitors on the IGF-I gene signature. We reasoned that regulation of genes by IGF-I (which made up the IGF gene signature) should be reversed by anti-IGF-IR inhibitors. Consistent with this, when we examined the levels of genes we previously found to be induced or repressed by IGF-I in neuroblastoma xenografts treated with an anti-IGF-IR (h10H5, Genentech) antibody (17), we found that there was a striking reversion in their levels (Fig. 4A). Thus, genes induced by IGF-I in MCF7 cells were repressed by h10H5 treatment of the neuroblastoma xenograft, and genes repressed by IGF-I were now induced. This result was highly significant (Fig 4D, Spearman's rank sum p~ 0). Highly similar results were obtained with an IGF-IR tyrosine kinase inhibitor (A-928605, Abbott) (18) that was administered *in vitro* to NIH3T3 fibroblasts transfected with the IGF-IR (Fig. 4B). Finally, we generated gene expression data from colon cancer xenografts (GEO) grown *in vitro* and then treated with an anti-IGF tyrosine kinase inhibitor BMS-754807 (12.5mg/kg/day) for various time-points of 1, 6 and 24 hours and 15 days. Treatment of these GEO xenografts resulted in gene expression values that were again reversed compared to the IGF-signature (Fig. 4C). Note that there was no change in the IGF signature after 1 hour, but after 6 hours of exposure to the drug a strong reversion of the IGF signature was seen, which is entirely consistent with the pharmacodynamics and pharmacokinetics of BMS-754807. **This data gives us confidence that our signature can measure IGF activity, and furthermore, suggests that the IGF signature might be a predictor of cells that have an active IGF pathway and thus may response to an IGF-IR inhibitor. Thus, the IGF-gene signature itself might be a marker of sensitivity and resistance to IGF-IR therapy.**



D

			IGF-induced (MCF-7)		IGF-repressed (MCF-7)	
Data source	**Model system**	**Inhibitor**	**Spearman's rank sum**	**P-value**	**Spearman's rank sum**	**P-value**
GSE11959	SK-N-AS xenografts	h10H5	-21.0	~0	16.6	~0
GSE14024	IGF1R-transfected 3T3 cultures	A-928605	-11.4	~0	5.4	<1E-7
unpublished	GEO xenografts	BMS-754807	-12.8	~0	12.5	~0

Figure 4: An IGF gene signature is reversed by treatment of cancer xenografts and IGF-IR overexpressing NIH3T3 fibroblasts with anti-IGF-IR inhibitors.
Genes in the IGF-I gene signature derived from MCF7 cells stimulated with IGF-I (12) were examined in **A**. a gene expression profile of neuroblastoma (SK-N-AS) xenografts treated with vehicle or an anti-IGF-R antibody and in **B**. IGF-IR transfected NIH3T3 fibroblasts treated with an IGF-IR small molecule inhibitor and in **C**. colon cancer xenografts treated with the small molecule inhibitor BMS-754807. For A-C, relative gene expression is represented using a yellow–blue color scale; patterns for genes that are upregulated (yellow) in the IGF gene signature are separate from the patterns for genes that are downregulated (blue). **D**. Statistical enrichment of the MCF7 IGF-I signature gene sets within each of the indicated IGF-I inhibitor treatment expression profiled datasets. All genes represented in the IGF-I inhibitor dataset were first ranked by expression in inhibitor/control (using two-sided t-test), then the overall positions of the MCF7 gene sets within the ranked lists was assessed by Spearman's rank sum (positive rank sum, associated with inhibitor group; negative rank sum, associated with control group). In all three datasets, treatment with IGF-IR inhibitors reversed expression of the IGF regulated genes.

To further identify markers of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor we used an unbiased transcriptomic approach by performing comparative gene expression analysis between sensitive and resistant breast cancer cell lines to BMS-754807 (19). Therefore, we first determined the sensitivity of BMS-754807 by MTS assay in a panel of 30 breast cancer cell lines. Among the different tumor cell lines, sensitivity presented as IC_{50} to BMS-754807 varied widely from 0.1µM to 25µM (Fig. 5). When defining cell lines as sensitive or resistant based on the median IC_{50} (6.4µM), we found a clear correlation between the sensitive/resistant classification to BMS-754807 and specific breast cancer subtypes. The greatest response to BMS-754807 was in basal-like/TNBC cell lines (10/15), while luminal

breast cancer cell lines and Her2 overexpressing cell lines were relatively resistant (13/15) (Fig 3). Compared to all cell lines, the TNBC group was enriched for cell lines with low IC_{50} ($p<0.01$, Spearman's rank sum, two-sided).

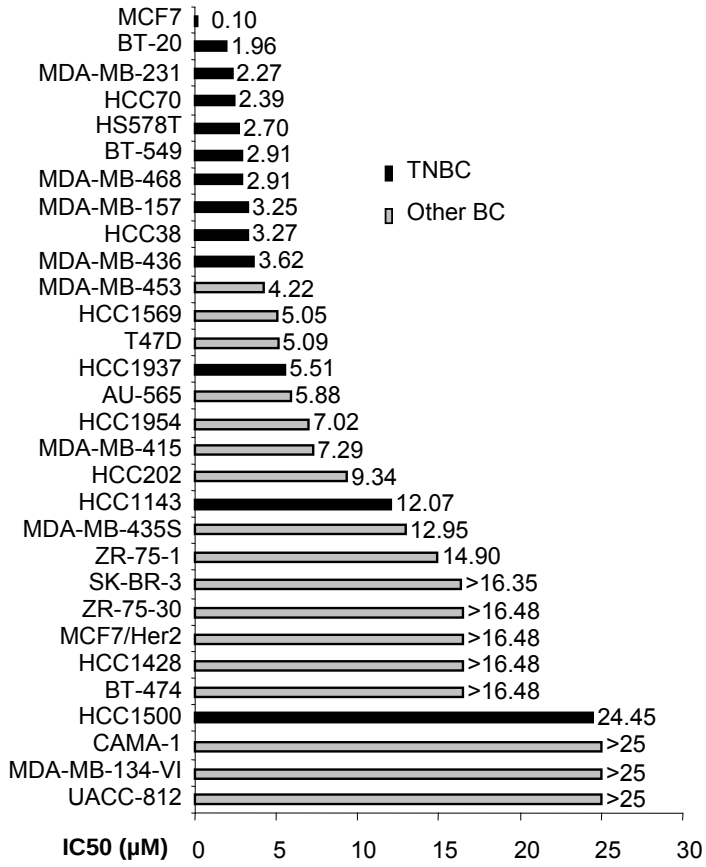


Figure 5: BMS-754807 is active in basal-like/triple-negative breast cancer cell lines. The concentration of BMS-754807 required to reduce growth by 50% (IC_{50}) was calculated for each cell line using monolayer proliferation and MTS assay. Breast cancer cells were seeded at 1,000 to 12,000 cells per well depending on the cell line in 96 well microtiter plates and incubated overnight. BMS-754807 was serially diluted and added. After 72 hr exposure, MTS assay was performed. Bars represent the average IC_{50} (µM) of each breast cancer cell line. For seven cells lines the IC_{50} was not reached (16.49µM and 25µM, respectively). Sensitive cell lines have an IC_{50} below the mean of the group of cells (6.4µM); resistant cell lines are above the mean. The graph shows cell lines ranked according to its IC_{50}. Black bars represent cell lines that have a basal-like gene expression signature (TNBC) based upon the studies of Neve et al (20) and grey bars represent luminal or HER2 positive cell lines.

We then performed comparative gene expression analysis between the ten most sensitive cell lines with an IC_{50} below 4µM and the 9 most resistant cell lines with an IC_{50} above 14µM BMS-754807 using gene expression data published by Neve et al (20). This analysis identified 136 probe sets corresponding to 114 genes ($p < 0.001$ and FDR < 5 %) that were differentially expressed between sensitive and resistant cell lines. The top 10 differentially expressed genes were validated by qRT-PCR in a panel of seven sensitive and six resistant breast cancer cell lines (Fig. 6). We identified that most of these 114 genes were actually markers of the basal or luminal subtype with sensitive cell lines expressing basal markers such as CAV1 and CAV2 whereas resistant cell lines expressed luminal markers such as ErbB3 and SPDEF.

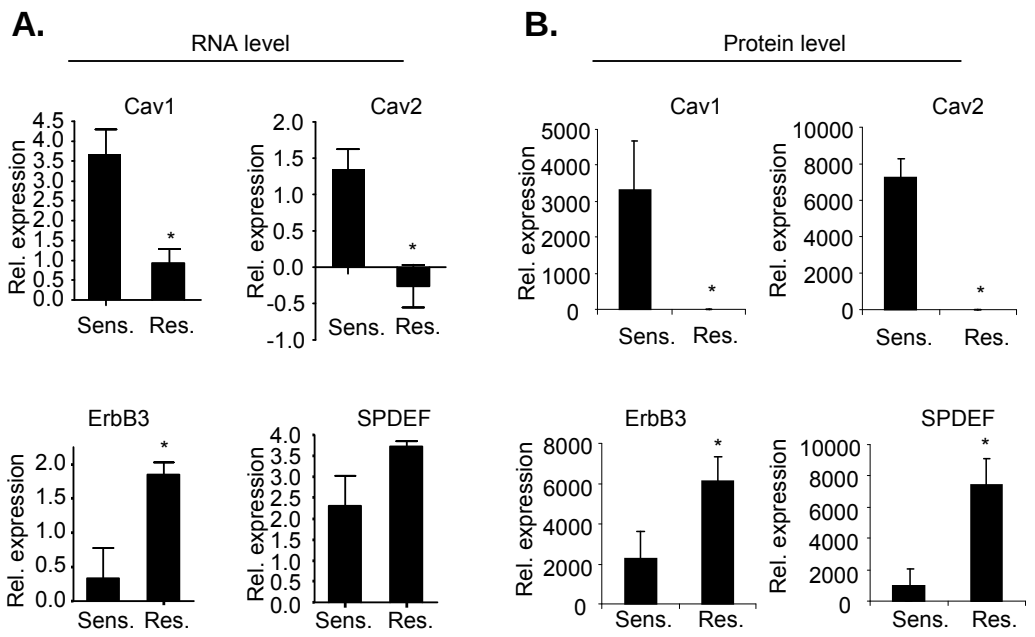


Figure 6: Sensitive cell lines express basal markers whereas resistant cell lines express luminal markers. A. The expression of differentially expressed genes were validated by qRT-PCR in a panel of seven sensitive cell lines (MCF7, BT20, MDA-MB-468, HS758T, MDA-MB-231, HCC38, MDA-MB-436) and six resistant cell lines (ZR75-1, SKBR3, BT474, CAMA-1, MDA-MB-134, UACC812). Analysis was done using the ΔΔCT method, normalizing first to the average of the housekeeping β-actin. The results are presented as log10 transformed transcript levels. Graphs represent the mean and error bars represent the SEM. Data points were compared by the two-tailed t-test. **B.** Protein quantification values for each cell line in A were taken from Neve et al. (20). Bars represent the mean and error bars represent the SEM. Data values were compared by the two-tailed t-test.

2. *Test whether response of cells to the IGF-IR inhibitor is correlated with markers identified in Aim 3.1*

In the previous aim 3.1 we identified markers of response to an IGF-IR tyrosine kinase inhibitor. Based on aim 3.1 we proposed that the IGF signature might be a predictor of cells that have an active IGF pathway and thus may response to an IGF-IR inhibitor. To test whether the presence of the IGF signature correlate with the response of cells to an IGF-IR inhibitor, we first examined the usefulness of the IGF gene signature in predicting patients who may respond to anti-IGF-IR therapy. Therefore, we studied cell lines grown in culture, where one can rapidly assess response and analyzed the IGF-I gene signature in a publicly available dataset of gene expression profiles from a large panel of breast cancer cell lines (20). Figure 7A shows a panel of breast cancer cell lines arranged according to their intrinsic subtype (as defined using the Hoadley dataset (21)). The IGF signature is present in the majority of basal-like (and TNBC) breast cancer cell lines. We assigned each cell line a t-score based upon the similarity of its gene expression profile to the IGF signature and the positive t-score in the majority of TNBC cell lines highlights the presence of the signature in this subtype (Fig. 7B). This data is entirely consistent with our previous report of the IGF signature in TNBC (12) suggesting that the IGF-IR pathway is highly active in the triple-negative/basal-like subtype of breast cancer.

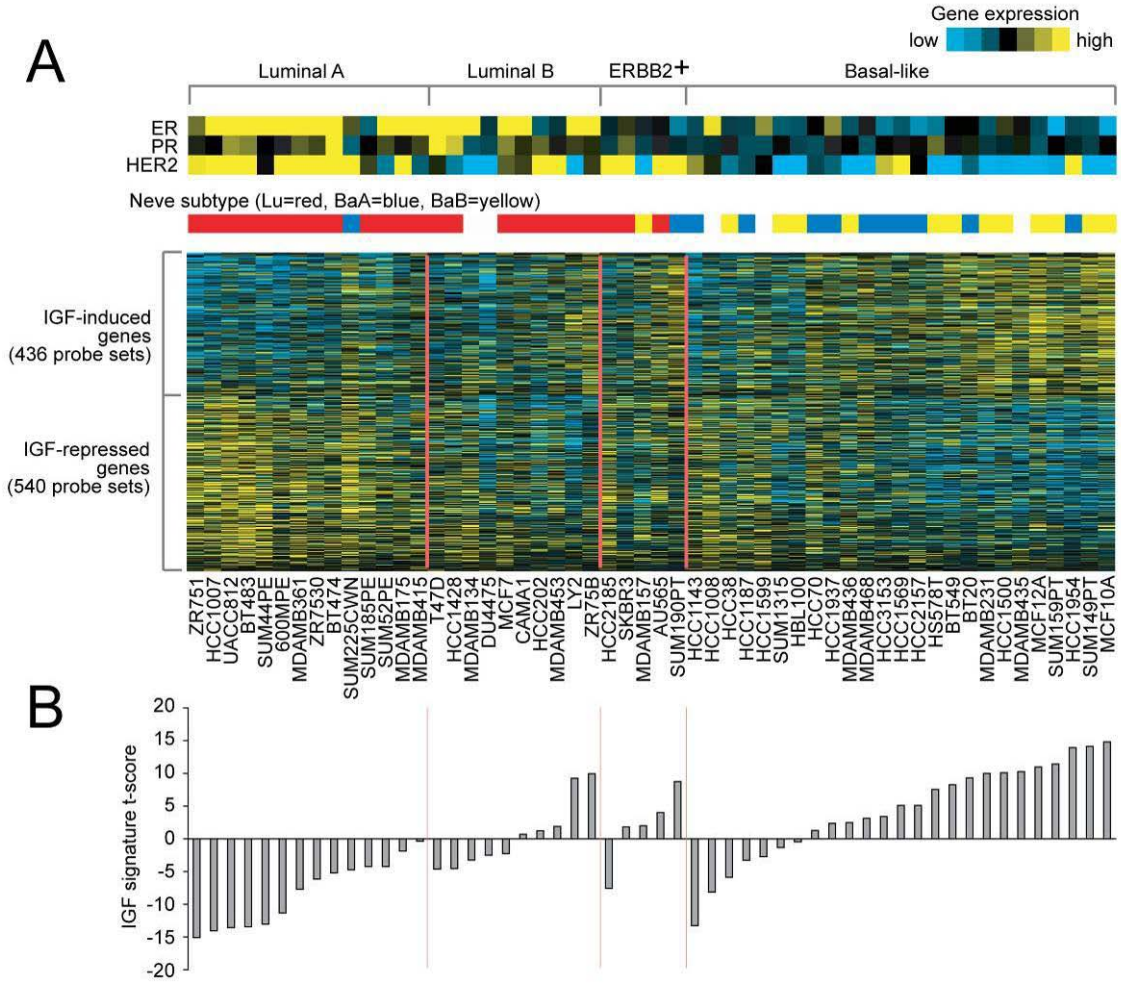


Figure 7: IGF gene signature is present in triple-negative/basal-like breast cancer cell lines. Breast cancer cell line profiles were classified using the Hoadley dataset (21), where intrinsic subtypes were previously defined. The correlation was computed between a given cell line gene expression profile and the mean centroid of each subtype; cell lines were then assigned to a subtype with the highest correlation. **A.** The heatmap represents the IGF gene signature in the cell lines according to subtype. ER, PR, and HER2 mRNA levels are shown. In addition, the breast cancer subtype is indicated as defined in the original gene expression study by Neve et al. **B**. The graph represents the t-score for each cell line based on the similarity to the IGF signature similar to previously published analyses (12). The t-score was defined as the two-sided t-statistic comparing the average of the IGF-induced genes with that of the IGF-repressed genes within each breast cancer cell line. The gene expression values in the breast cancer cell line dataset (n=51 breast cancer cell lines, Neve et al.(20)) were first normalized to the median before computing the t-score. For each gene transcription profile dataset, we assigned intrinsic molecular subtypes to the cell lines, essentially as previously described (22), using the human tumor dataset from Hoadley et al. (21) to define the subtype-specific expression patterns.

To test whether the IGF signature is a marker of sensitivity and resistance to an IGF-IR tyrosine kinase inhibitor BMS-754807 we plotted the t-score for the IGF gene signature for each cell line against the IC_{50} (as determined in aim 3.1). There was a significant inverse correlation ($r=-0.41$, $p=0.014$), with a higher t-score (indicating an active IGF pathway) being associated

with a greater response (lower IC_{50}) to BMS-754807 (Fig. 8). **These data confirms that the response of cells to BMS-754807 correlates with the IGF-gene signature. In addition, this analysis highlights that BMS-754807 is specifically active in TNBC.**

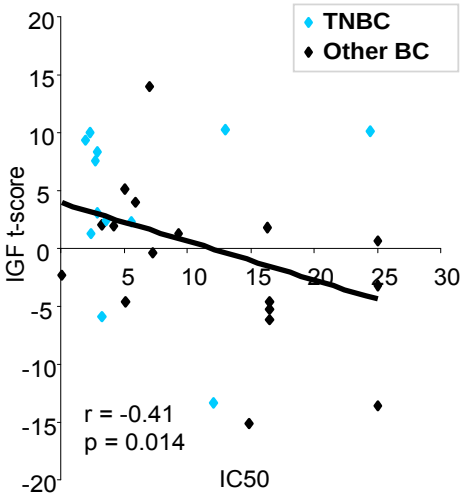


Figure 8: The IGF-I gene signature correlates with response to BMS-754807. The IC_{50} is plotted alongside the t-score for the IGF signature for each cell line. Pearson correlation shows there is a significant correlation (r=-0.41, p =0.014), with a higher t-score (indicating an active IGF-IR pathway) being associated with a greater response (lower IC_{50}) to BMS-754807. This data also highlights that BMS-754807 is active in basal-like/triple-negative breast cancer cell lines.

In the unbiased approach of aim 3.1 we identified 114 genes that were differentially expressed between sensitive and resistant cell lines. We found that most of these 114 genes are markers of the basal or luminal subtype with sensitive cell lines expressing basal markers such as CAV1 and CAV2 whereas resistant cell lines expressed luminal markers such as ErbB3 and SPDEF. To proof that these markers correlate to the response of cells to the IGF-IR inhibitor BMS-754807 we next analyzed the 114 genes in a panel of 51 breast cancer cell line profiles with known and unknown IC_{50} to BMS-754807 (20). Hierarchical clustering separated the cells lines into two major bins: ER-negative cell lines that were sensitive and ER-positive cell lines that were mostly resistant (Fig. 9).

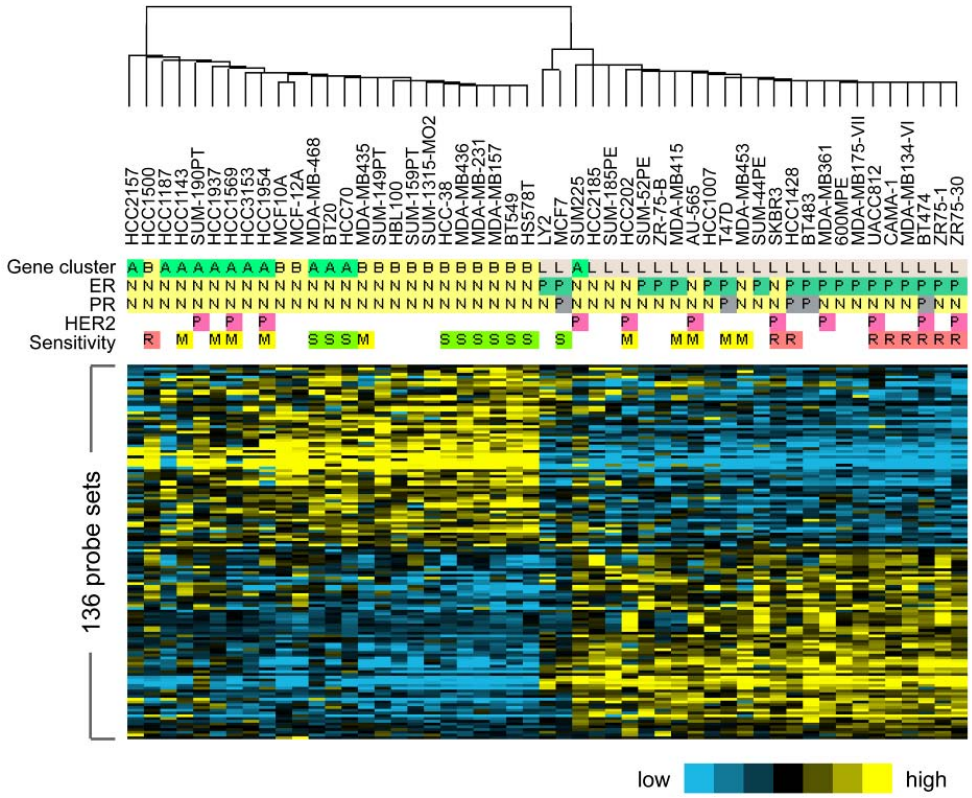


Figure 9. 114 differentially expressed genes identify triple negative breast cancer cell lines as most responsive to BMS-754807. 136 differentially expressed probe sets, representing 114 genes, were analyzed in 51 breast cancer cell line profiles published by Neve *et.al*. with known and unknown IC_{50}. S=sensitive (IC_{50}<4µM), M=Medium IC_{50}: (4µM<14µM), R=Resistant (IC_{50}>14µM). In addition, the ER, PR and Her2 status is indicated (P = positive, N = negative). Gene-cluster indicates the breast cancer subtype as defined in the original gene expression study by Neve *et al*. (A = basal A, B = basal B, L = Luminal).

To validate this clustering data, we determined the sensitivity to BMS-754807 of breast cancer cell lines with an unknown IC_{50} by MTS-assay. Among the cell lines tested the TNBC cell lines SUM149PT and MCF10A showed the greatest response to BMS-754807 whereas cell lines that are ER negative but overexpress Her2 (SUM225 and SUM190PT) are less sensitive to BMS-754807 (Fig. 8 and Table 1). Luminal breast cancer cell lines such as ZR75B, MDA-MB-175VII and MDA-MB-316 showed the least response to BMS-754807. **In summary, these experiments showed that 114 genes are markers that correlate with response of cells to the IGF-IR inhibitor BMS-754807. In addition, we could confirm that BMS-754807 is especially active in TNBC cell lines.**

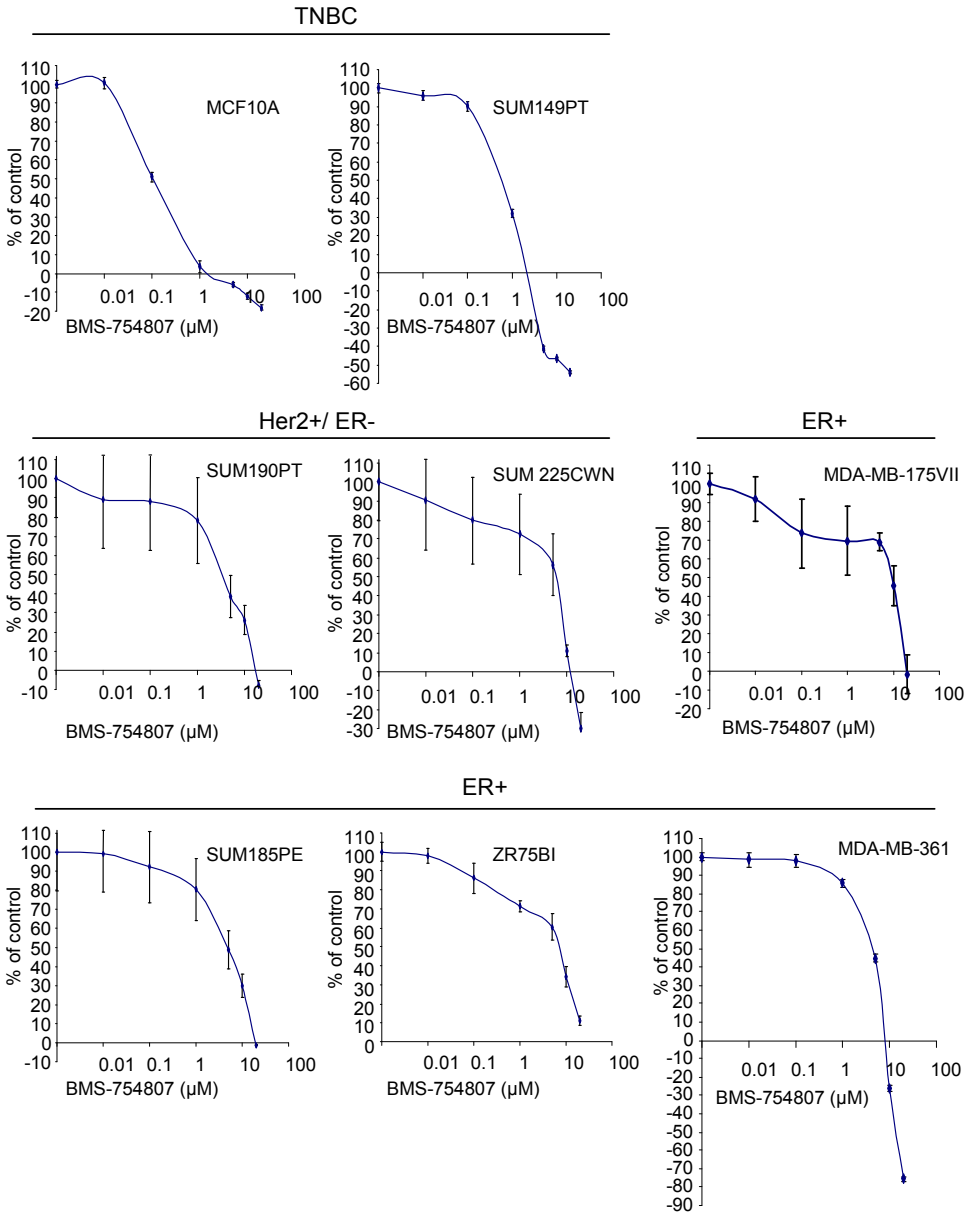


Figure 10. BMS-754807 is especially active in TNBC cell lines
Breast cancer cells with unknown IC_{50}s to BMS-754807 were plated in 96 well microtiter plates and incubated overnight. BMS-754807 was serially diluted and added. After 72 hr exposure, MTS assay was performed. Sensitive cell lines have an IC_{50}<4μM, moderate IC_{50} between 4 μM and 14μM, resistant: IC_{50}>14μM

Table 1. Summary of the effect of BMS-754807 on cells lines with an unknown IC_{50}

Cell lines	Response to BMS-754807	ER status	Breast Cancer subtype
SUM149PT	sensitive	-	TN
MCF10A	Sensitive	-	TN
SUM225CWN	Moderate	-	(HER2+)
Sum190PT	Moderate	-	(HER2+)
SUM185PE	Moderate	Low +	Luminal
ZR75B	Moderate	+	Luminal
MDA-MB-175VII	Moderate	+	Luminal
MDA-MB-361	Moderate	+	Luminal

3. Examine if an IGF-IR inhibitor is effective in breast cancer xenografts identified as having an active IGF-IR pathway

Preclinical cancer research has relied heavily upon cell lines grown in culture and then xenografted for growth in mice. However, recent work has shown that human cancers placed directly into the mouse (tumorgrafts) maybe a more appropriate model that is better at predicting response to drugs in humans (23). We have recently developed several new tumorgraft models of human TNBC. We screened seven TNBC tumorgrafts for activity of the IGF-IR by both immunohistochemistry (IHC) and immunoblotting (IB). Figure 11A shows three representative tumorgrafts expressed various levels of IGF-IR and had divergent levels of active phosphorylated pY-IGF-IR. We generated gene expression data from all of the tumorgrafts and calculated an IGF signature t-score for each tumorgraft. Analysis of these profiles showed that tumorgraft MC1 (24) had the highest level of IGF-IR and pY-IGF-IR, and in addition had a high IGF signature t-score. Protein lysates from the same tumorgrafts as in Fig 11A confirmed that MC1 had the highest activation of IGF-IR (Fig. 11B). Activation of downstream signaling molecules varied among the tumorgraft models. MC1 had high levels of IRS1 and activated AKT whereas the tumorgraft 2665A showed activation of MAPK.

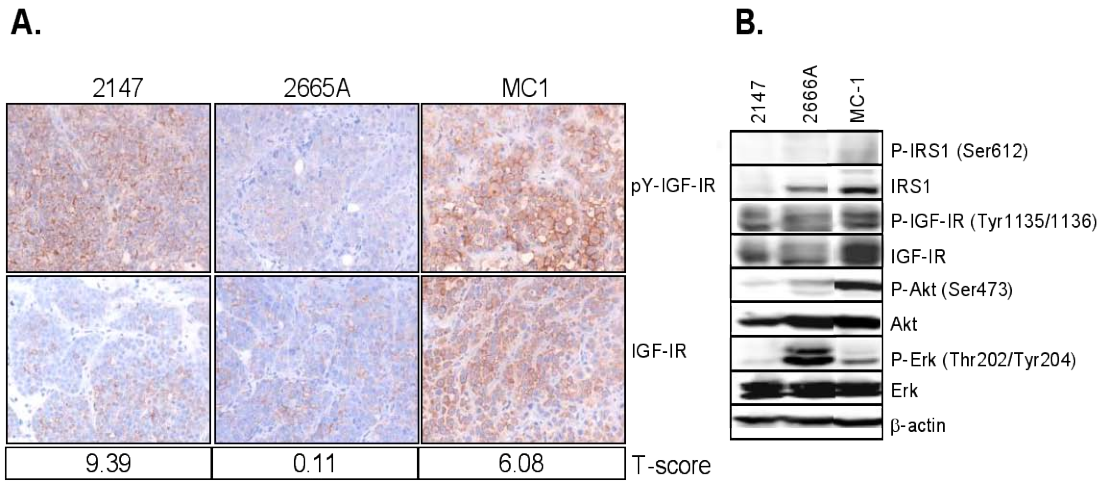


Figure 11. TNBC human tumorgrafts express high levels of IGF-IR
A. Three different TNBC human tumorgraft lines (2147, 2665A, MC1) were harvested and processed in paraffin. Immunohistochemistry was performed for anti-pY-IGF-IR (Phospho-IGF-IR) and total IGF-IR. Microarray analysis was performed on these tumorgrafts. Based on the gene expression data an IGF signature t-score was calculated for each tumorgraft as indicated underneath the representative picture. **B.** The same three tumorgraft lines were lysed and analyzed by immunoblot and probed using IGF-IR specific antibodies as well as total/phospho-specific antibodies for IRS1, AKT, ERK1/2. ß-actin was used as a loading control. Tumorgraft lines that were not relevant to this study were cropped out between 2665A and MC1.

Our studies in Aim 3.1 and 3.2 identified TNBC cell lines as having an active IGF pathway. As we previously showed that BMS-754807 is most active in TNBC cell lines *in vitro* (Aim 3.1 and 3.2), we directly examined the effectiveness of the IGF-IR inhibitor BMS-754807 in the MC1 tumorgraft model of TNBC breast cancer alone as a single agent or in the presence of chemotherapy (docetaxel). We chose the tumorgraft MC1 for this preclinical study as it showed the highest levels of active and total IGF-IR and also a high IGF t-score (Figure 11). A recent

study examining timing of anti-IGF-IR therapy and chemotherapy in cells in culture showed that most efficacious combination was chemotherapy followed by anti-IGF-IR therapy (25). We thus administered docetaxel followed by BMS-754807 the next day. Single agent BMS-754807 achieved a statistically significant ($p<0.001$) reduction in tumor growth when compared with the control group (Fig. 12A). Docetaxel stabilized MC1 tumor growth. Strikingly, combined treatment with BMS-754807 and docetaxel showed superior tumor growth inhibition to either single agent alone ($p<0.001$), and four out of six mice receiving the combined agents had tumors regress until no tumor was palpable.

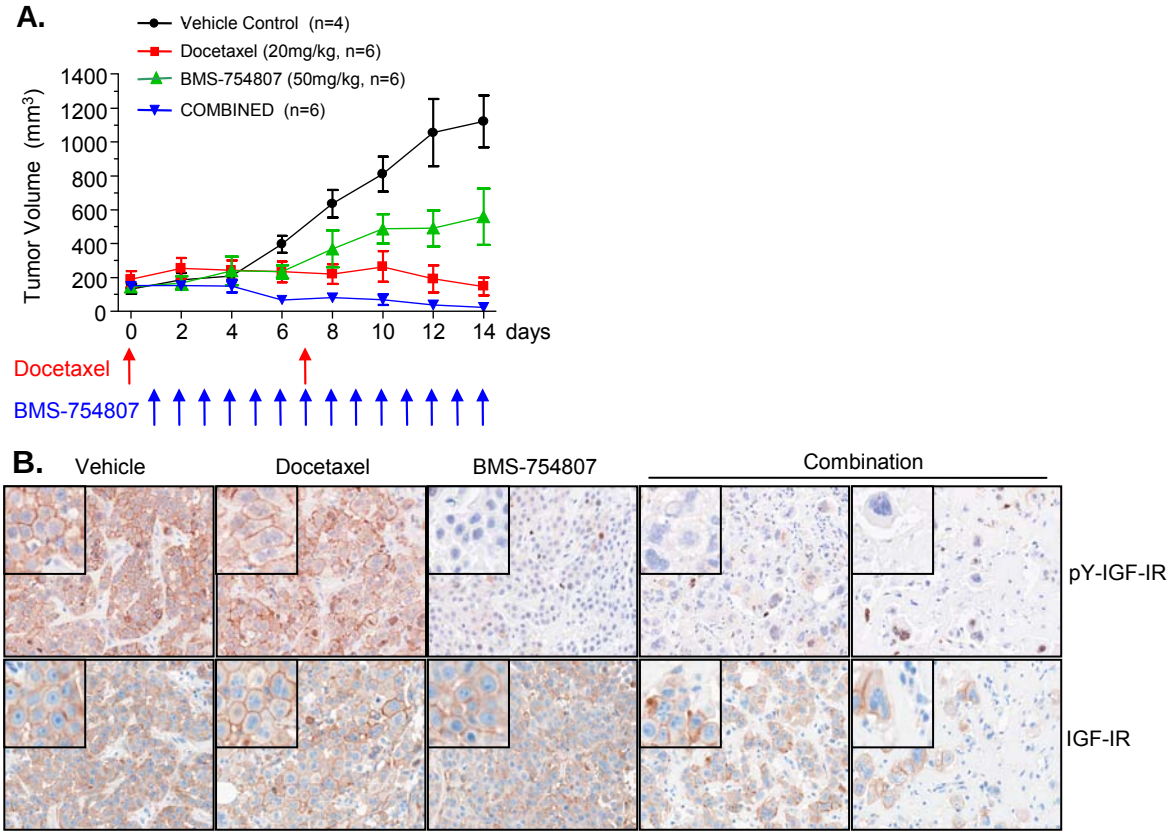


Figure 12. BMS-754807 inhibits growth of TNBC tumografts and causes regression in combination with chemotherapy

A. 4 to 6-week-old female NOD/scid mice were transplanted with a 1mm^3 piece of tumorgraft into a cleared number four mammary fat pad. When tumors reached a volume between 100-200mm^3 they were randomized to receive the following treatments: vehicle, 50mg/kg BMS-754807 daily by oral gavauge, 20mg/kg Docetaxel weekly by intraperitoneal injection or the combination of 50mg/kg BMS-754807 daily and 20mg/kg docetaxel weekly by intraperitoneal injection. Tumor volume and body weight was measured daily. All mice were sacrificed when control tumors reached 1000mm^3. **B.** Untreated and treated tumors were processed in a tissue microarray (TMA) for immunohistochemical analysis. Immunohistochemistry was performed for Phospho and total IGF-IR. Representative IHC staining of the treatment groups is taken at 40x magnification.

To confirm the ability of BMS-754807 to inhibit IGF-IR activity in triple negative breast cancer, tyrosine phosphorylation and total level of IGF-IR was examined in the various treatment groups. Both p-IGF-IR and IGF-IR showed membrane staining with a small amount of cytoplasmic staining (Fig. 10B). There was no change in the levels of pY-IGF-IR between tumors treated with docetaxel or vehicle (Fig. 12B). In contrast, BMS-754807 completely blocked IGF-IR phosphorylation. There was no change in levels of total IGF-IR between the different treatment groups.

We next analyzed the histology of the tumors among the different treatment groups. Several reports have shown that chemotherapy agents such as docetaxel affect the stability of the microtubules and in doing so induce mitotic catastrophe (26, 27). Mitotic catastrophe results from aberrant mitosis, or missegregation of chromosomes followed by cell division which results in the formation of multinucleated giant cells leading to cell death. Cell death through mitotic catastrophe may occur through apoptosis as well as necrosis (27). MC1 tumorgrafts treated with Docetaxel showed relatively few multinucleated giant cells (Fig. 11A). However, the addition of BMS-754807 dramatically increased docetaxel-induced mitotic catastrophe (Fig. 13A). Analysis of H&E staining showed that normal breast cancer cells were still present in Docetaxel treated tumors whereas only multinucleated cells were present in the combination treated tumors.

Since multinucleated cells may be temporarily viable and mitotic catastrophe may be a process leading to death (27), we analyzed treated tumors for replication, proliferation, and apoptosis. All treatment groups showed a reduction in replication as indicated by less BrdU incorporation into DNA compared to untreated tumor cells (Fig. 13B). BMS-754807 alone reduced replication by 36% (22% BrdU positive cells in vehicle tumors versus 16% in treated tumors) but this was not significant (adjusted p-value=0.13). Chemotherapy significantly reduced replication by 55% (adjusted p-value=0.015) and the combination by 59% compared to vehicle (adjusted p-value=0.0018). We next analyzed proliferation as assessed by Ki67 positive cells (Fig. 13 C and E). Whereas both single agents alone were able to significantly reduce proliferation by 30-32% (adjusted p-values < 0.0001), combination treatment resulted in only a 17% reduction in proliferation (adjusted p-value < 0.005, compared to vehicle). We then investigated whether single agents alone or the combination of BMS-754807 and Docetaxel induces cell death. BMS-754807 caused a 4-fold elevation in apoptosis as measured by cleaved caspase 3 (CC3) from 3% apoptotic cells in vehicle treated tumors to 11% in BMS-754807 treated tumors (Fig. 13D and E), however this elevation was not significant (adjusted p-value = 0.36). Chemotherapy resulted in a 6.7-fold induction of apoptosis compared to vehicle (adjusted p-value=0.1) which again wasn't significant, whereas the combination caused a striking 12-fold induction of apoptosis (Fig. 13D and E) which was highly significant (adjusted p-value=0.005). In addition, combination therapy resulted in massive cell destruction through necrosis as shown in Figure 13A.

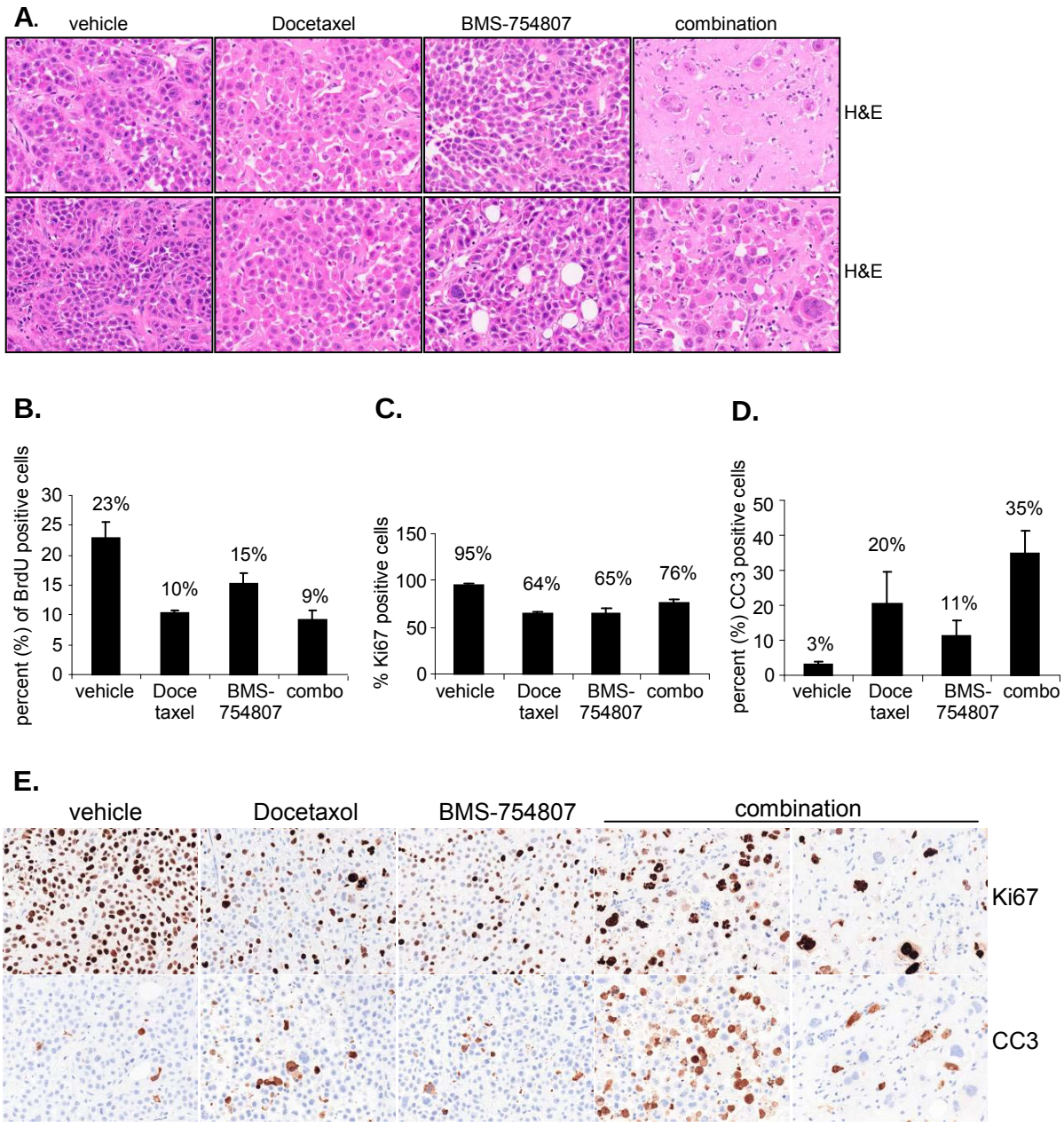


Figure 13. BMS-754807 sensitizes MC1 tumorgrafts to docetaxel induced mitotic catastrophe
A. Representative tumor sections of the treatment groups stained with hematoxylin and eosin are taken at 40x magnification. **B.** Quantification of BrdU incorporation per cell was done by image analysis. Data represents means ± SE of 12 representative pictures per treatment group. **C and D.** The percentage of Ki67/CC3-positive cells within the tumor was scored. Values represent the means ± SE of 12 representative pictures per treatment group. **E.** Representative tumor sections of the treatment groups stained with Ki67 and CC3 (cleaved caspase 3) are taken at 40x magnification.

KEY RESEARCH ACCOMPLISHMENTS

Aim 1

- Showed that overexpression of IRS2 has no affect upon ErbB2-induced tumorigenesis and metastasis in transgenic mice
- Revealed that IRS2 doesn't co-immunoprecipitate with ErbB2

Aim 2

- Tested and optimized RPPA system for IGF-I stimulation of breast cancer cells
- Used RPPA to measure the effect of IGF-I and insulin in 23 breast cancer cell lines
- Discovered that cell lines harbor 3 general responses to ligand stimulation
- Developed computer simulations that predicted the effect of IGF-I stimulation on breast cancer cells
- Used computer simulations to show that IRS-1 is a key signaling node that needs to be inhibited in breast cancer.

Aim 3

- Used bioinformatics and public datasets to show that an IGF-gene signature can measure IGF activity, as based on reversion of the signature in three different cancer models treated with different anti-IGF-IR therapies.
- Showed that the IGF-IR tyrosine kinase inhibitor BMS-754807 inhibited proliferation in a panel of breast cancer cell lines with selective activity in triple negative breast cancer cell lines.
- Comparative gene expression analysis among the most sensitive and resistant cell lines to BMS-754807 identified 114 genes which confirmed TNBC as being sensitive to BMS-754807.
- Showed that the IGF signature in present in triple-negative/basal-like breast cancer cell lines.
- Revealed that sensitivity of cells to BMS-754807 correlates with the IGF-gene signature which highlights that BMS-754807 is especially active in TNBC cell lines.
- Finally, new tumorgraft models of TNBC show expression and activation of IGF-IR and treatment with BMS-754807 in combination with chemotherapy results in complete tumor regression.

REPORTABLE OUTCOMES

- **Beate C. Litzenburger**, Chad J. Creighton, Anna Tsimelzon, Bonita T. Chan, Susan G. Hilsenbeck, Fei Huang, Joan M. Carboni, Marco M. Gottardis, Jenny C. Chang, Michael T. Lewis, and Adrian V. Lee. High IGF-IR activity in triple-negative breast cancer cell lines correlates with sensitivity to IGF-IR inhibitor BMS-754807. (32nd Annual CTRC-AACR San Antonio Breast Cancer Symposium, #1132, 2009, SABCS Basic Science Scholarship)

- **Beate C. Litzenburger**, Chad J. Creighton, Anna Tsimelzon, Bonita T. Chan, Susan G. Hilsenbeck, Fei Huang, Joan M. Carboni, Marco M. Gottardis, Jenny C. Chang, Michael T. Lewis, and Adrian V. Lee. High IGF-IR activity in triple-negative breast cancer cell lines

correlates with sensitivity to IGF-IR inhibitor BMS-754807. (Dan Duncan Cancer Center Symposium, 2009)

- **Litzenburger BC**, Creighton CJ, Tsimelzon A, Chan BT, Hilsenbeck SG, Wang T, Carboni JM, Gottardis MM, Huang F, Chang, Lewis MT, Rimawi MF, Lee AV. High IGF-IR activity in triple-negative breast cancer cell lines and tumorgrafts correlates with sensitivity to anti-IGF-IR therapy. *In revision* Clin Cancer Res 2010

- Defended and was awarded a Ph.D. (Magna Cum Laude).

- Applied for and received a position at Grunenthal Group, a large pharmaceutical company

CONCLUSION

In Aim 1 I focused on the role of IRS2 in ErbB2-mediated tumorigenesis in transgenic mice. This study was based upon an exciting proteomic study which suggested that there might be an interaction. However, we found that overexpression of IRS2 didn't affect ErbB2-induced tumorigenesis and metastasis. Therefore, I expanded the research project to other receptors such as IGF-IR for which IRSs serve as the main adapter proteins for downstream signaling. In Aim 2 I examined IGF signaling in a panel of breast cancer cell lines to correlate levels of IRS with response to IGF-I and insulin. We used reverse phase proteomic arrays to develop proteomic profiles of both insulin and IGF-I action in 23 breast cancer cell lines. Computer simulations of this data identified IRS1 as a critical signaling node which needs to be inhibited in breast cancer cells. In Aim 3 I expanded the study outside of IRSs to identify biomarkers for response to IGF-IR and subsequent sensitivity to an IGF-IR small tyrosine kinase inhibitor. Originally, the laboratory previously developed the IGF signature to learn more about transcriptional events downstream of IGF-IR, and to examine the role of IGF-IR regulated genes in breast cancer. However, the obvious question as to the potential role of the IGF signature in predicting response to anti-IGF-IR therapy arose. We showed here that the IGF signature can measure IGF activity in tumors and that the IGF signature weakly correlated (r=0.41) with response of cells to an IGF-IR inhibitor. The strength of the signature may come when used in combination with IGF-IR protein levels and activity as potential marker for response to IGF-IR therapy. Therefore, IGF-IR alone, or the IGF signature alone maybe insufficient to indicate an active IGF pathway, but the combination may yield better prediction. To this end, we used this strategy to select a TNBC tumorgraft for study and found dramatic effects of an IGF-IR inhibitor. Further comprehensive studies are required to definitively prove whether the combination of IGF-IR levels and downstream gene transcripts is a useful method for identify patients who may respond to anti-IGF-IR therapy.

I have been extremely fortunate to obtain pre-doctoral training in an outstanding environment at Baylor College of Medicine. I hope that my studies will continue the movement to targeting IGF-IR in breast cancer, and I fully expect that IRSs will continue to develop as critical biomarkers for response to IGF-IR inhibitors.

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APPENDIX

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Beate Litzenburger	**Research Manager**
eRA COMMONS USER NAME LITZENBU	

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
RWTH Aachen, Aachen, Germany	M.Sc.	2000-2006	Molecular and Cellular Biology
Baylor College of Medicine, Houston, TX	Ph.D.	2006-2010	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE:

06/2010-present Grunenthal GmbH, Aachen, Germany
Project Manager: Pain medicine

12/2006-06/2010 Baylor College of Medicine, Houston, TX, USA
Lester and Sue Smith Breast Center, Predoctoral fellow
PhD Thesis

10/2005-07/2006 Baylor College of Medicine, Houston, TX, USA
Lester and Sue Smith Breast Center, Master's student
Master Thesis: Characterization of a small molecule inhibitor in the treatment of breast cancer

06/2005-09/2005 Grunenthal GmbH, Aachen, Germany
Research & Development, Intern
Project: Planning and monitoring of a clinical phase II study

01/2004-06/2004 Baylor College of Medicine, Houston, TX, USA
Department of Molecular and Cellular Biology, Intern
RNA-interference as a gene therapy strategy for the treatment of Epidermolysis Bullosa Simplex

2003/2004 Medical Center 'Blondelst.' Aachen, Germany
Assistant Medical Technician
Analysis of human serum of HIV patients using Flow Cytometry

HONORS AND AWARDS

2009	San Antonio Breast Cancer Symposium Basic Science Scholarship
2008-present	Department of Defense Predoctoral Traineeship Award
2007-2008	Fellowship of the German Academic Exchange Service (DAAD)
2007	1st place oral presentation award winner at the Breast Center Retreat, Baylor College of Medicine, Houston, TX
2006	1st place poster award winner at the Breast Center Retreat, Baylor College of Medicine, Houston, TX
1999-2000	President's Honor Roll. Butler County Community College, Kansas, USA

PUBLICATIONS

Litzenburger BC, Creighton CJ, Tsimelzon A, Chan BT, Hilsenbeck SG, Wang T, Carboni JM, Gottardis MM, Huang F, Chang, Lewis MT, Rimawi MF, Lee AV. High IGF-IR activity in triple-negative breast cancer cell lines and tumorgrafts correlates with sensitivity to anti-IGF-IR therapy. *In revision* Clin Cancer Res 2010

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POSTER PRESENTATIONS

Beate C. Litzenburger, Chad J. Creighton, Anna T simelzon, Bonita T. Chan, Susan G. Hilsenbeck, Fei Huang, Joan M. Carboni, Marco M. Gottardis, Jenny C. Chang, Michael T. Lewis, and Adrian V. Lee. High IGF-IR activity in triple-negative breast cancer cell lines correlates with sensitivity to IGF-IR inhibitor BMS-754807. (32nd Annual CTRC-AACR San Antonio Breast Cancer Symposium, #1132, 2009, SABCS Basic Science Scholarship)

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